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Research Article

SACCHAROMONOSPORA OCEANI VJDS-3, A POTENT ACTINOBACTERIAL STRAIN FROM MANGROVE ECOSYSTEM

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ABSTRACT

Objective: The aim of the present study was to isolate, identify, and analyze the phylogenetic characteristics of a rare actinobacterial strain VJDS-3 with antagonistic activities isolated from Mangrove ecosystems of Nizampatnam, Guntur District, Andhra Pradesh, India.

Methods: Soil samples collected were pre-treated with calcium carbonate and used for isolation of potent actinobacterial strain designated as VJDS-3. Identification of the strain was carried out by studying the micro-morphological, cultural, biochemical, and physiological methods. The phylogenetic study of the strain was carried out by employing 16S rDNA sequence-based analysis. The phylogenetic tree was constructed using the Molecular Evolutionary Genetic Analysis software version 6.

Results: The potent actinobacterial strain was identified as *Saccharomonospora oceani* VJDS-3, and the bioactive metabolites produced by the strain inhibited Gram-positive bacteria (*Bacillus megaterium*, *Bacillus subtilis*), Gram-negative bacteria (*Xanthomonas campestris, Pseudomonas aeruginosa, Proteus vulgaris,* and *Escherichia coli*), and fungi (*Aspergillus niger, Botrytis cinerea, Fusarium solani, Fusarium Oxysporum,* and *Candida albicans*).

Conclusion: The results of the experiment showed that the crude ethyl acetate extract of *S. oceani* VJDS-3 showed significant antimicrobial potential, and hence it can be used for isolation of compounds with pharmaceutical importance.

Keywords: Mangrove ecosystems, Phylogenetic study, *Saccharomonospora oceani* VJDS-3, Bioactive compounds.

INTRODUCTION

Microbial products are attractive sources of chemotherapeutic agents, justifying industrial interest in modern biotechnology. Actinomycetes are widely distributed in natural and man-made environments which play an important role in the degradation of organic matter. They are the rich source of antibiotics and bioactive molecules and are of considerable importance in industry. With conventional isolation techniques applied, many of the isolates recovered on agar plates belong to genus *Streptomyces*, which are the dominant actinomycetes in soil [1-3]. Several factors must be considered to screen novel bioactive molecules: Choice of screening source, pre-treatment, selective medium, cultural conditions, and recognition of rare candidate colonies on a primary isolation plate. The role of rare actinomycetes as sources of novel bioactive leads became apparent [4]. Rare actinomycetes are strained whose isolation frequency by conventional methods is much lower than that of streptomycete strains and a basic knowledge of the habitat, physiology, and productivity of molecules of rare actinomycetes gradually increased.

Employing pre-treatments of soil by drying and heating stimulated the isolation of rare actinomycetes as the spores of most actinomycete genera were found to resist desiccation and heat [5]. An alternative approach was to make the isolation procedure more selective by adding chemicals such as phenol to the soil suspension [6,7]. Specialized growth media were developed for isolating rare actinomycete genera by supplementing with macromolecules such as casein, chitin, hair hydrolysate, and humic acid chosen as carbon and nitrogen sources [8-10]. Diagnosis of isolates on a primary isolation plate and recognition as a novel taxa was very important both for practical and taxonomical purposes.

In the present investigation, an attempt has been made to isolate rare actinobacteria with antimicrobial potential from the Mangrove soils of Nizampatnam, Guntur district and study their taxonomic characteristics.

METHODS

Sampling and pre-treatment of soil

Soil samples were randomly collected from different locations of mangrove ecosystem of Nizampatnam, Guntur district, south coast of Andhra Pradesh, India. Samples were collected from 6 cm to 10 cm depth and brought to the laboratory in sterilized containers and air-dried at room temperature. The air-dried soil sample was pretreated with calcium carbonate (10:1 w/w) and incubated at 37°C for 4 days [11].

Selective isolation of actinobacteria

The pretreated soil samples were serially diluted in sterile distilled water and plated on selective media such as humic acid vitamin (HV) agar medium supplemented with 3% NaCl [12]. The medium was adjusted to pH 7.0 and 0.1 mL of diluted sample was spread over HV agar supplemented with 25 µg/mL nystatin and 25 µg/mL streptomycin to reduce the fungal and bacterial contamination, respectively, and incubated at 30±2°C for 3 weeks. Actinobacterial colonies [13] were picked out, purified and preserved on yeast extract malt extract dextrose (YMD) agar slants at 4°C [14]. The isolated actinobacterial strains were then screened for their potential to generate bioactive compounds (Atta *et al*., 2009).

Identification of the rare actinobacterial strain

Morphological, cultural, physiological, and biochemical characteristics of the strain

The rare actinobacterial strain was characterized by cultural, morphological, physiological, biochemical, and molecular methods. The microscopic characterization was carried out by slide culture method [15] taking into account the nature of mycelium, color, and spore arrangement [16]. The morphological characteristics were assessed using scanning electron microscopy (SEM: Model- JOELJSM 5600, Japan) of 6-day-old culture grown on yeast extract malt extract dextrose agar (YMD) medium at various magnifications. The strain was grown on seven International Streptomyces Project (ISP) media

and four non-ISP media to observe the cultural characteristics such as color of aerial mycelium, substrate mycelium, pigment production, and spore formation [17]. Melanin pigment production was assessed by culturing the strain on tyrosine agar (ISP-7) medium [18]. Hydrolysis of starch and nitrate reduction [19] and H_2S production were also tested [20]. Physiological characteristics such as the effect of pH (5-9), temperature (20-45°C), and salinity on the growth of the strain were analyzed. The susceptibility of the strain to different antibiotics was also determined by paper disc method [21].

Molecular identification

The genomic DNA used for the polymerase chain reaction (PCR) was prepared from the colonies grown on YMD agar for 3 days. The total genomic DNA extracted from the strain was isolated by employing the DNA purification Kit (Pure Fast® Bacterial Genomic DNA purification kit, Helini Bio molecules, India) according to the manufacturer protocol. The 16S rRNA gene fragment was amplified using Actino specific forward primer -5'-GCCTAACACATGCAAGTCGA-3' and actino specific reverse primer - 5'-CGTATTACCGCGGCTGCTGG-3'. Conditions of the PCR were standardized with initial denaturation at 94°C for 3 minutes followed by 30 cycles of amplification (Denaturation at 94°C for 60 seconds, annealing temperature of 55°C for 60 seconds, and extension at 72°C for 60 seconds) and an addition of 5 minutes at 72°C as final extension. The amplification reactions were carried with a total volume of 50 µL in a gradient PCR (Eppendorf, Germany). Each reaction mixture contained 1 µL of DNA, 1 µL of 10 *P* mol forward 16S actino specific primer (5'-AAATGGAGGAAGGTGGGGAT-'3), 1 µL of 10 pmol reverse 16S actino specific primer (5'- AGGAGGTGATCCAACCGCA-'3), 25 µL of master mix, and 22 µL of molecular grade nuclease free water. The separation was carried out at 90 Volts for 40 minutes in TAE buffer with 5 µL of ethidium bromide. PCR product was analyzed using 1% agarose gel, and the fragment was purified (Helini Pure Fast PCR clean up kit, Helini Bio molecules, India) as per the manufacturer's instructions. The bands were analyzed under UV light and documented using Gel Doc. The direct sequencing of PCR products was performed by dideoxy chain termination method using 3100-Avant genetic analyzer (Applied Bio systems, USA).

Pair wise sequence alignment

The gene sequence of the strain was aligned using BLAST against the gene library available for *Saccharomonospora* species in the NCBI and the GenBank. Pairwise evolutionary distances were computed by MEGA-6 software.

Multiple sequence alignment

The phylogenetic analysis was conducted using the maximum parsimony method of the strain using BLAST and CLUSTAL W. The closely related homologous strains were identified, retrieved and compared to the sequence of the isolated strain using CLUSTAL W available with the MEGA 6 Version [22].

Nucleotide sequence accession numbers

The 16S rRNA gene (rDNA) sequence of the strain VJDS-3 is registered in the GenBank database.

Growth pattern of the strain VJDS-3

For determination of growth pattern, the strain was inoculated into 250 ml flasks containing 100 ml YMD broth and incubated at 30±2°C on a rotary shaker at 180 rpm. The flasks were harvested at 24 hrs interval, and the growth of the strain was determined by taking the dry weight of biomass. The culture filtrates obtained after separating the biomass were extracted with ethyl acetate, and antimicrobial activity of the crude extract was determined by agar well diffusion method.

Extraction of metabolites and antimicrobial assay

The antimicrobial activity of the strain was determined by agar well diffusion assay. The homogenous culture suspension prepared by suspending 3-day-old culture in sterile saline was used to inoculate YMD broth (seed medium), and the culture was incubated at 30°C for 48 hrs on a rotator shaker at 180 rpm. Seed culture at the rate of 10% was transferred to YMD broth (fermentation medium). The fermentation was carried out at 30±2°C for 120 hrs under agitation at 180 rpm. The antimicrobial compound was recovered from the filtrate by solvent extraction method. Ethyl acetate was added to the filtrate (1:1) and shaken vigorously. The ethyl acetate extract was evaporated to dryness in water bath and the residue thus obtained was used to determine antimicrobial activity. Ethyl acetate itself was used as negative control. About 80 µl of the crude extract and 80 µl of negative control were poured into separate wells. The standard antibiotic disc was placed on the agar surface as a positive control. For each bacterial strain, controls were maintained utilizing pure solvent. Plates were incubated at 37°C for 48 hrs, and inhibition zones (in mm) were measured after 24-48 hrs. The experiment was carried out in triplicates for each test organism, and the mean values were computed.

Test organisms

Bacteria: *Staphylococcus aureus* (MTCC 3160), *Lactobacillus casei* (MTCC 1423), *Bacillus subtilis* (ATCC 6633), *Bacillus megaterium* (NCIM 2187), *Shigella flexneri* (MTCC 1457), *Xanthomonas campestris* (MTCC 2286), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 9027), and *Escherichia coli* (ATCC 9027).

Fungi: *Aspergillus niger, Botrytis cinerea, Fusarium solani Fusarium Oxysporum,* and *Candida albicans* (MTCC 183).

RESULTS AND DISCUSSION

Among the 20 actinobacterial strains isolated from the mangrove ecosystem of nizampatnam, the predominant actinobacterial strain VJDS-3 was found to be potent and exhibited strong antimicrobial activity against Gram-positive, Gram-negative bacteria, and fungi. The strain VJDS-3 exhibited typical morphological characteristics of the genus *Saccharomonospora.* Morphological and micro-morphological observation of the strain revealed that the strain produced non-fragmenting substrate mycelium. No sporangia are formed. The strain produced single warty spores oval to round in shape formed on very short sporophores.

Identification of the strain VJDS-3

The cultural characteristics of the strain are represented in Table 1. The strain VJDS-3 exhibited good growth on tryptone yeast extract agar (ISP-1), YMD agar (ISP-2), glycerol asparagines agar (ISP-5), and starch casein salts agar (non-ISP). The growth was moderate on nutrient agar (non-ISP). Poor growth was observed on czapek-dox agar. The color of aerial mycelium was white and substrate mycelium was pale yellow on the different media tested. Soluble pigment production was not observed on the media tested. As physiological and biochemical characteristics are significant tools for identification of Actinobacteria, which influence the growth rate [20-22], several tests were conducted for identifying strain VJDS-3 (Table 2). The strain VJDS-3 exhibited a positive response to catalase production. The pH range of the strain is between 5 and 10 with the optimum being 7, and the range of temperature for growth was 28-30°C with the optimum being 28°C. Sodium chloride tolerance of the strain was also studied as the salt concentration has a profound effect on the production of antibiotics by microorganisms. The strain could grow well in the medium supplemented with 3% sodium chloride and shown tolerance up to 9%. Though the strain VJDS-3 utilized a wide range of carbon sources, maltose, sucrose, D-glucose, and galactose supported good growth of the strain. The strain was resistant to the majority of antibiotics tested and showed sensitivity to penicillin, co-trimoxazole, erythromycin, and cefuroxime.

Molecular characterization

The 16S rDNA sequence data supported the assignment of the strain VJDS-3 to the genus *Saccharomonospora* and species *oceani.* The partial 16S rDNA sequence of the strain VJDS-3 was submitted to the GenBank

Table 1: Cultural characteristics of the strain VJDS-3

Name of the medium	Growth	AM*	SM**	Pigmentation
Tryptone yeast extract agar (ISP-1)	Good	White	Pale yellow	No
Yeast extract malt extract dextrose agar (ISP-2)	Good	White	Pale yellow	No
Oat-meal agar (ISP-3)	-	-	-	No
Inorganic salts Starch Agar (ISP-4)	-	-	-	No
Glycerol Asparagine agar (ISP-5)	Good	White	Pale yellow	No
Tyrosine agar (ISP-7)	-	-	-	No
Czapek-Dox agar	Poor	Ash	Pale yellow	No
Nutrient agar	Moderate	White	Pale yellow	No
Bennet's agar	-	-	-	No
Starch casein salts agar	Good	White	Pale yellow	No
Glucose tryptone agar	Good	White	Pale yellow	No

*Aerial mycelium, **Substrate mycelium. -: No growth, ISP: International Streptomyces Project

Table 2: Morphological, biochemical, and physiological characteristics of the strain VJDS-3

Character	Response
Morphological characters	
Sporophore morphology	Warty
Color of aerial mycelium	White
Color of substrate mycelium	Pale yellow
Biochemical characters	
Catalase production	+
Urease production	-
Hydrogen sulfide production	-
Nitrate reduction	-
Starch hydrolysis	+
Gelatin liquefaction	+
Methyl red test	-
Voges-Proskauer test	-
Indole production	-
Citrate utilization	-
Physiological characters	
Gram reaction	+
Production of melanin pigment	-
Range of temperature for growth	28-30°C
Optimum temperature for growth	28°C
Range of pH for growth	5-10
Optimum pH for growth	7
NaCl tolerance	Up to 9%
Utilization of carbon sources (w/v)*	
Lactose	+
Maltose	+++
Raffinose	+
Sucrose	+++
Arabinose	++
D-glucose	+++
Galactose	+++
Fructose	++
Starch	+
Mannitol	+
Cellulose	++
Antibiotic sensitivity	
Gentamicin (10 µg)	R
Kanamycin (10 µg)	R
Penicillin (10 µg)	S
Co-trimoxazole (25 µg)	S
Ciprofloxacin (10 µg)	R
Erythromycin (10 µg	S
Lincomycin (10 µg)	R
Cefuraxime (30 µg)	S

*Growth of the strain measured as dry weight of the mycelium, +++: Good growth, ++: Moderate growth, +: Weak growth, -: Negative/no growth, S: Sensitive, R: Resistant, P: Positive, N: Negative

database under an accession number KP231373. The partial sequence was aligned and compared with all the 16S rDNA gene sequence available in the GenBank database using the multi-sequence advanced BLAST comparison tool. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W program from the MEGA 6 Version. Phylogenetic tree (Fig. 1) was constructed using MEGA software Version 6 using maximum parsimony method.

Table 3: Antibacterial and antifungal activity of *Saccharomonospora oceani* VJDS-3

Test organism	Zone of inhibition (mm)	Positive control[#]
Bacteria		
Staphylococcus aureus	18±0.15	22±0.05
Lactobacillus casei	20±0.08	28±0.10
Xanthomonas campestris	21±0.12	25±0.12
Bacillus megaterium	25±0.14	25±0.08
Bacillus subtilis	22±0.08	26±0.09
Shigella flexneri	20±0.09	22±0.06
Escherichia coli	14±0.09	20±0.05
Pseudomonas aeruginosa	19±0.11	22±0.15
Proteus vulgaris	16±0.11	26±0.08
Fungi		
Aspergillus niger	14±0.05	20±0.05
Botrytis cinerea	13±0.08	28±0.08
Fusarium solani	18±0.12	21±0.05
Fusarium oxysporum	14±0.12	18±0.12
Candida albicans	22±0.05	28±0.05

[#]Positive control: Tetracycline against bacteria, Griseofulvin against yeast and Carbendazim against fungi. Values are mean±S.E.M (n = 3)

Growth pattern and antimicrobial profile of *Saccharomonospora oceani* VJDS-3

The growth curve and antimicrobial profile of *S. oceani* were studied at regular intervals for up to 8 days in batch culture. The stationary phase of *S. oceani* VJDS-3 extended from 168 hrs to 216 hrs of incubation. The secondary metabolites obtained from 8-day-old culture showed high antimicrobial activity against the test microbes (Fig. 2). However, the 7-day culture extracts of *Saccharomonospora halophila* and *Saccharomonospora paurometabolica* isolated from Algerian Sahara soils exhibited strong antimicrobial activity against Gram-negative bacteria [23]. Munaganti *et al*. (2015) noted the production of antimicrobial metabolites from 5-day-old culture of *Rhodococcus erythropolis* VL-RK-05 [24]. Naragani *et al*. (2014) reported that 5-day old-culture extracts of *Streptomyces violaceoruber* VLK-4 evidenced the production of antimicrobial compounds [25]. The secondary metabolites obtained from 4-day-old culture of *Nocardia levis* MK-VL-113 isolated from laterite soils of Guntur showed high antimicrobial activity against the test microbes [26].

The antimicrobial spectrum of the strain cultured on YMD broth for 11 days was shown in Table 3. The metabolites extracted from the 8-day-old culture broth showed maximum activity against *B. megaterium*, *B. subtilis*, *X. campestris,* and *S. flexneri*. In case of fungi, *C. albicans* showed high sensitivity when compared to the other fungi tested.

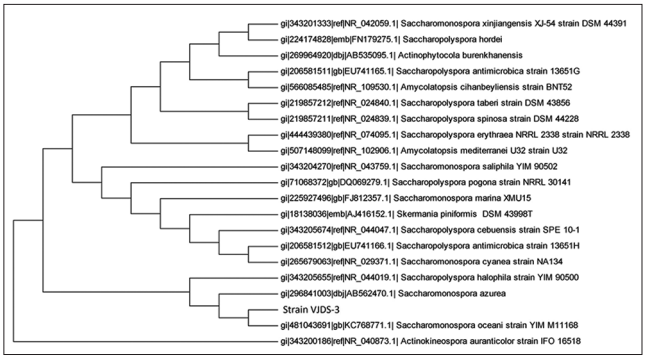

Fig. 1: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Saccharomonospora* strain VJDS-3 and related members of the genus *Saccharomonospora*

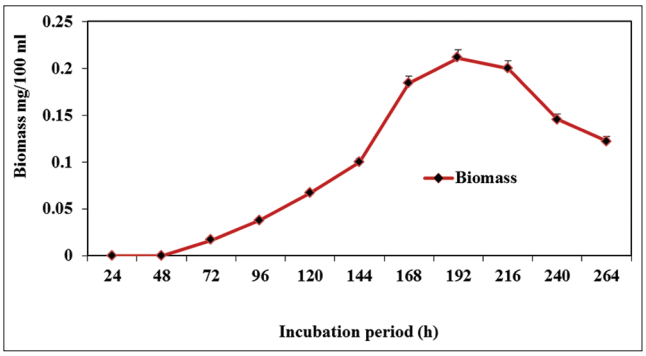

Fig. 2: Growth pattern of the strain *Saccharomonospora oceani* VJDS-3

CONCLUSION

The present investigation highlights the antimicrobial potential of *S. oceani* VJDS-3. Further study on optimization, purification, and chemical characterization of bioactive compounds of the strain are in progress.

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